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Intracellular biochemical manipulation of phototransduction in detached rod outer segments

(retinal rod photoreceptor/signal transduction/second messengers/transducin)

W. A. SATHER AND P. B. DETWILER†

University of Washington, Department of Physiology and Biophysics, SJ-40, Seattle, WA 98195

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ABSTRACT **Recent progress in understanding phototransduction has come primarily from studies on cell-free systems. To investigate the transduction process under physiological conditions, a fully functional preparation of retinal rod outer segments without attached inner segments was developed that allows electrical recording of light-sensitive current during intracellular dialysis with defined solutions. No light-sensitive current is recorded from detached outer segments dialyzed with nucleotide-free solutions, whereas cells detached from the retina into Ringer's solution containing 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor) develop a light-sensitive inward dark current. This indicates that there is a basal level of cGMP-specific phosphodiesterase activity in the dark. Detached outer segments dialyzed with ≥20 μM cGMP rapidly develop a light-suppressible current. A current of similar magnitude is generated more slowly during dialysis with a 50-fold greater concentration of GTP. Apparently, cGMP can be synthesized from GTP by guanylate cyclase in the outer segment. Cells dialyzed with cGMP alone show a reduced light sensitivity that is restored to normal by addition of 20 μM GTP. This action of GTP is antagonized by guanosine 5′-[β-thio]diphosphate. These findings are in good agreement with biochemical evidence indicating that a GTP-binding protein (transducin) plays a pivotal role in the generation of responses to light. The recovery of photocurrent following a brief flash is delayed or abolished by dialysis with solutions that lack ATP or contain guanosine 5′-[γ-thio]triphosphate, a nonhydrolyzable GTP analog. These results support the view that both GTP hydrolysis by activated transducin and ATP-dependent phosphorylation of a rhodopsin photoproduct are necessary for termination of the transduction process.**

In retinal rods of vertebrates, light reduces a standing dark current that flows into the outer segment through light-regulated cation channels and out of the inner segment through a light-insensitive conductance. Suppression of the dark current is the end point of the transduction process that couples the absorption of light to an electrical signal. Cation channels in the outer segment surface membrane are opened by cGMP (1–5). By triggering photoisomerization of rhodopsin, light stimulates a cGMP-specific phosphodiesterase (PDE) resulting in cGMP hydrolysis and channel closure (reviewed in refs. 6–8). Biochemical studies indicate that two intermediates couple photoisomerization to PDE stimulation. The first intermediate, a photoproduct of rhodopsin (Rh*), activates the second, a guanine nucleotide-binding (G) protein (G_t, often called transducin), by catalyzing the exchange of GTP for GDP bound to the α subunit of G_t ($G_{t\alpha}$). The activated form of the G protein ($G_{t\alpha}$·GTP) stimulates PDE by removing an inhibitory constraint. Termination of the transduction cascade requires the inactivation of both intermediates. This is believed to be accomplished through the phosphorylation of Rh*, by rhodopsin kinase, and the hydrolysis of bound GTP, by the intrinsic GTPase activity of $G_{t\alpha}$. While studies on cell-free systems have provided extensive evidence to support this mechanism, it has not been tested directly in physiological experiments. We have developed a fully functional preparation of intact rod outer segments that allows electrical recording of light responses during internal dialysis. With this method we have obtained evidence in support of key steps in the above transduction scheme.

MATERIALS AND METHODS

Recordings were obtained from mechanically isolated rod outer segments from retinas of dark-adapted lizards (*Gekko gekko*). Dissection of the eye, dissociation of the retina, and all subsequent manipulations were performed in darkness, using infrared illumination and image converters. After a patch pipette was sealed onto a selected outer segment, the pipette potential was clamped at −20 mV. Whole-cell currents were recorded using conventional electronics [List EPC-7 amplifier (List Electronic, Darmstadt, F.R.G.)].

Nucleotides were added to a standard internal solution containing 92 mM potassium aspartate, 7 mM NaCl, 2.2 mM $MgCl_2$, and 2.8 mM Hepes at pH 7.4. Divalent cations were buffered with EGTA and/or EDTA. Their free concentrations were calculated from published dissociation constants and took into account the Ca^{2+} and Mg^{2+} contamination of stock salt solutions and distilled water, measured by atomic absorption spectroscopy. Nucleoside triphosphates were added with equimolar $MgCl_2$ and the osmolality of the final solution was adjusted to 290 ± 15 mosmol/kg by addition of sucrose. GTP, ATP, and cGMP were obtained from Sigma, and guanosine 5′-[γ-thio]triphosphate and 5′-[β-thio]diphosphate (GTP[γS] and GDP[βS]) were from Boehringer Mannheim. HPLC was used to check the purity of nucleotide solutions. The external solution was a mixture of equal volumes of lizard Ringer's solution and amphibian culture medium (GIBCO) yielding the following concentrations of relevant ions: 122 mM Na^+, 4 mM K^+, 1.0 mM Ca^{2+}, 1.2 mM Mg^{2+}, 116 mM Cl^-, 10 mM HCO_3^-, 1.2 mM SO_4^{2-}, 1.4 mM Hepes, and pH 7.4.

RESULTS

Yau and Nakatani (9) found that perfusion of an open-ended outer segment with a solution containing cGMP opens cation channels and gives rise to membrane current that can be rendered light-sensitive by addition of GTP. This demon-

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Abbreviations: PDE, phosphodiesterase; Rh*, rhodopsin photoproduct; G protein, guanine nucleotide-binding protein; G_t, the G protein of rod outer segments (also called transducin); $G_{t\alpha}$, α subunit of G_t; GTP[γS], guanosine 5′-[γ-thio]triphosphate; GDP[βS], guanosine 5′-[β-thio]diphosphate; iBuMeXan, 3-isobutyl-1-methylxanthine.

†To whom reprint requests should be addressed.

strates that some aspects of normal function can be restored to a photoreceptor fragment by providing the appropriate biochemical constituents. We used a similar approach to study the conditions that support generation of light-sensitive current in intact rod outer segments. The composition of outer segment cytoplasm was manipulated by diffusional exchange with a patch-pipette solution during whole-cell recording. In order to avoid complications arising from biochemical and electrical activity in the inner segment, the experiments were performed on outer segments that were not connected to an inner segment. Gigaohm seals on detached outer segments were obtained with patch pipettes filled with a standard internal solution containing various amounts of cGMP, GTP, and ATP. Disruption of the patch membrane to provide direct communication between the pipette and the cell interior was signaled by an abrupt increase in capacitance. The records in Fig. 1 show breakthrough (arrow) from cell-attached to whole-cell recording, development of dark current, and the effect of flashes or steps of 520-nm light. Fig. 1 *A–E* present typical recordings obtained with five different

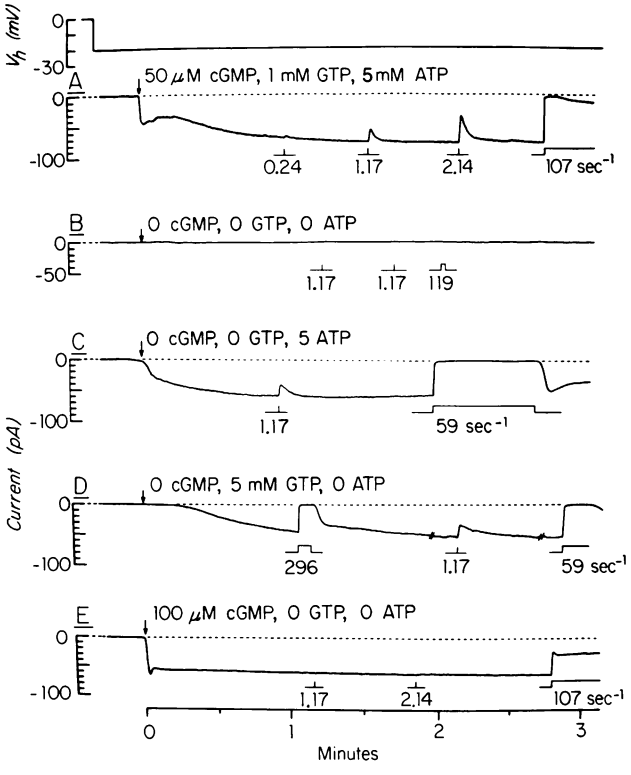

FIG. 1. Whole-cell recording with five different pipette solutions. The pipette potential was set at −20 mV (V_{hold}, top trace), and breakthrough from cell-attached to whole-cell recording is indicated (arrow). (*A*) The chart record shows the development of dark current and its suppression by 20-msec flashes and a step of light during the first 3 min of recording with a pipette containing 50 μM cGMP, 5 mM ATP, 1 mM GTP, and 20 μM EGTA in standard internal solution. (*B–E*) Currents recorded from other cells with no nucleotides (*B*), only ATP (*C*), only GTP (*D*), and only cGMP (*E*). No current developed in the absence of nucleotides (*B*). Either ATP or GTP alone was able to support a light-sensitive dark current (*C* and *D*). The onset of dark current was slower in recordings with pipette solutions containing only nucleoside triphosphates than in recordings with solutions containing cGMP. There was no consistent difference, however, between the rate of current development in recordings with ATP versus GTP. The light monitor under each trace gives the intensity of 520-nm light in photons per μm^2 per flash or per second for steps. The internal solution used for *B–E* contained 220 μM EGTA (pCa 8.9, pMg 2.66). Time since the onset of whole-cell recording is the same for all traces and is indicated by the scale at the bottom. The two breaks in *D* are 10- and 30-sec dark gaps. Temperature was 16–18°C.

pipette solutions. In *A*, the pipette solution contained 50 μM cGMP, 5 mM ATP, and 1 mM GTP. As we reported previously (10, 11), under these conditions cation channels in the surface membrane open and carry an inward dark current that is suppressed by light (see also refs. 12 and 13). The light sensitivity, reversal potential, and noise characteristics of photocurrents recorded in this way are similar to those recorded from intact cells. Over the course of a typical 45- to 75-min recording, there is a slowing of response kinetics and some loss of light sensitivity. For this reason, whenever possible, results obtained with different pipette solutions are compared during the first few minutes of the recording or at equivalent later times.

In the absence of nucleotides, light-regulated channels in detached rod outer segments are closed (Fig. 1*B* and Table 1) (11). This is not due to intracellular accumulation of divalent cations, because no dark current appears even if 10 mM EDTA is added to the pipette solution (Table 1). However, large dark currents (168 ± 20 pA, n = 4) are recorded even with zero-nucleotide pipette solutions when outer segments are detached in external solution containing 1 mM 3-isobutyl-1-methylxanthine (iBuMeXan), a membrane-permeant PDE inhibitor. Exposure to iBuMeXan apparently prevents the destruction of a pool of cGMP. Since animals were dark-adapted for several hours before an experiment and all subsequent manipulations were performed using only infrared illumination, we conclude that there is a significant level of PDE activity in the dark (14, 15). During the time it takes to prepare the outer segment and obtain a whole-cell recording (15–25 min) the dark activity of PDE is sufficient to hydrolyze the outer segment's store of cGMP, causing channels to close. A PDE activity in darkness could account for the continuous low-frequency (0.01–0.1 Hz) fluctuations in dark current that have been observed in detached outer segments (10, 11) as well as intact rods (16, 17). If PDE activation were due to thermal isomerization of rhodopsin, then the frequency spectrum of the dark fluctuations would be identical to the frequency spectrum of the single-photon response (17). That the spectra do not match (unpublished data) suggests that if basal PDE activity is responsible for the fluctuations in dark current, it arises from spontaneous activation of intermediate steps in transduction rather than thermal isomerization of rhodopsin.

Millimolar concentrations of either ATP or GTP can support a light-sensitive dark current (Fig. 1 *C* and *D*). Since neither GTP nor ATP is known to open cation channels in excised patches (1), we attribute the gradual development of dark current in these recordings to the synthesis of cGMP in the outer segment. Although guanylate cyclase is required for cGMP synthesis, the location of the enzyme within rods has not been determined (6). Our data provide strong evidence that guanylate cyclase is present in the outer segment and that cGMP can be synthesized from an exogenous supply of GTP.

Table 1. Relationship between cGMP and dark-current (I_{dark}) amplitude

cGMP, μM	I_{dark}, pA [mean ± SEM (n)]		
	220 μM EGTA (pCa 8.9, pMg 2.66)	10 mM EDTA (pCa 11.1, pMg 6.25)	1 mM iBuMeXan (pCa 8.9, pMg 2.66)
0	0.9 ± 0.4 (13)	7.2 ± 5.3 (5)	—
20	6.0 ± 1.2 (8)	—	29.6 ± 5.9 (11)
50	29.4 ± 3.5 (16)	165.3 ± 28.9 (3)	—
100	86.7 ± 19.2 (6)	—	—

Steady-state dark current was recorded with pipette solutions containing no GTP or ATP, the indicated concentration of cGMP, and either 220 μM EGTA, or 10 mM EDTA, or 220 μM EGTA plus 1 mM iBuMeXan in standard internal solution. V_{hold} = −20 mV.

We conclude that ATP is able to generate dark current by providing high-energy phosphate for the regeneration of GTP through the transphosphorylation of guanosine phosphates. The possibility that either ATP or GTP may donate phosphate for the synthesis of the other nucleoside triphosphate makes it important that experiments designed to investigate the actions of one nucleotide be carried out using minimal concentrations of the other.

Dark-current amplitude is graded with the concentration of cGMP in the pipette solution. The dose–response relation for cGMP in the absence of nucleoside triphosphates is presented in Table 1. The average current recorded with 20 μM cGMP is only 6 pA, less than 1% of the maximum. This suggests that intact outer segments are much less sensitive to applied cGMP than are excised patches, which have a K_m in the vicinity of 10 μM (3, 4, 9). The sensitivity of the outer segment to cGMP is enhanced ≈5-fold by adding 1 mM iBuMeXan to the pipette solution; internal solutions that contained iBuMeXan but no cGMP did not give rise to dark current. These results provide further evidence that PDE is active in the dark. By hydrolyzing a portion of the cGMP that enters the outer segment from the patch pipette, the basal activity of PDE shifts the apparent dose–response curve for cGMP to the right and may account for the difference in the sensitivity of intact outer segments and excised patches.

Dark current is supported by micromolar amounts of cGMP alone, but it is relatively insensitive to light. This can be appreciated by comparing the light responses in Fig. 1*E* with those evoked by equal intensity flashes in Fig. 1 *A*, *C*, and *D*. Data from other cells indicate that after 3 min of recording with a pipette solution containing 50 μM cGMP and no ATP or GTP, a flash of moderate intensity (2.1 photons per μm^2) reduced the dark current by 13 ± 3% (n = 3). For a control solution containing the same concentrations of cGMP (50 μM) and divalent cations (10 mM EDTA: pCa 10.5, pMg 5.4) but supplemented with 500 μM ATP and 500 μM GTP, a flash of the same intensity blocked 49 ± 2% (n = 4) of the dark current. In the absence of ATP and GTP there is further decline in light sensitivity with time. After 30–40 min of recording with 50 μM cGMP solution, dark currents are not suppressed by steps of unattenuated white light (1.5×10^6 photons per μm^2 per sec). The dramatic loss of light sensitivity can be prevented by the addition of 20 μM GTP but not by 20 μM ATP. The effect of GTP on photocurrent light sensitivity confirms earlier work by Yau and Nakatani (9), who used truncated rods. It is also consistent with biochemical studies that indicate that activation of a G protein (G_t) by GDP/GTP exchange is essential for transduction. The inability of 20 μM ATP to substitute for 20 μM GTP indicates that, although the outer segment can synthesize GTP from ATP, this is not an efficient process with micromolar ATP concentrations.

Tests for the involvement of a G protein were conducted with guanine nucleotide analogs. The control trace (Fig. 2*A*), recorded in the presence of GTP, shows responses to four flashes of progressively higher intensity. The recovery of the light response is rapid and may include a transient undershoot of the baseline dark current. In comparison, the recovery of responses recorded in the presence of GTP[γS] (Fig. 2*B*) is incomplete (18). Similar results were observed in nine experiments on detached outer segments. In all cases a brief flash evoked a response that rose rapidly to a peak followed by partial recovery to a maintained plateau. The amount of partial recovery decreased with each subsequent flash. Responses recorded in the presence of GDP[βS] recover fully but have reduced light sensitivity. Fig. 2*C* shows two sequential intensity series. The most intense stimulus delivered 25,000 photons per μm^2 yet failed to completely suppress the dark current; in control solution saturation was reached with 10 times less light. Responses evoked by flashes of equal

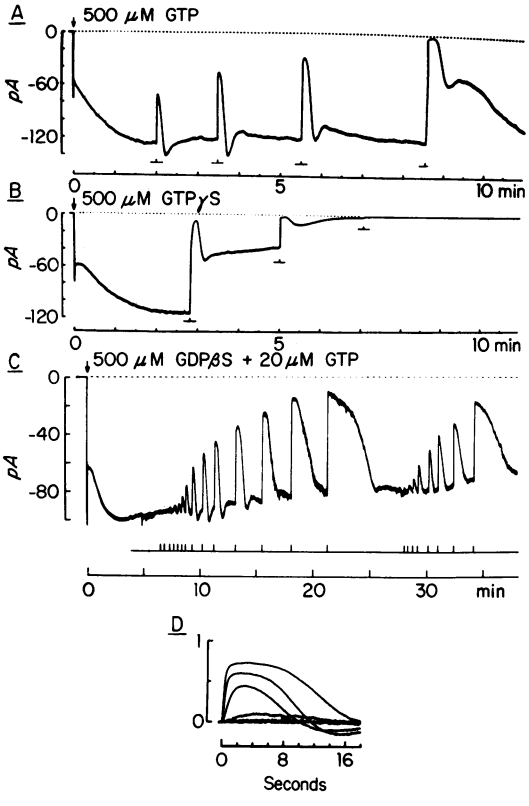

FIG. 2. Effect of guanine nucleotide analogs. Continuous chart records from three different cells, showing development of dark current and light responses during whole-cell recording with pipette solutions containing GTP (*A*), GTP[γS] (*B*), and GDP[βS] (*C*). (*A*) Current recording with control pipette solution (containing 50 μM cGMP, 500 μM ATP, and 500 μM GTP) shows a family of responses to flashes delivering 1.2, 12, 58, and 1400 photons per μm^2. Light monitor and time since onset of whole-cell recording are indicated by line segments and scales below each chart record. (*B*) In the presence of GTP[γS] (pipette solution containing 50 μM cGMP, 500 μM ATP, and 500 μM GTP[γS]), the light response rises but does not recover fully. The current recording shows the response to sequential flashes (58 photons per μm^2). (*C*) Responses recorded with 500 mM GDP[βS] in a pipette solution containing 50 μM cGMP, 500 μM ATP, and 20 μM GTP recover normally but have reduced light sensitivity. The current trace shows two sequential response families. The first used a series of 14 progressively brighter flashes starting with 1.2 and ending with 25,000 photons per μm^2. The second response family was evoked by a series of 8 flashes starting with 23 and ending with 12,500 photons per μm^2. The first three flashes are the same intensity as the first three flashes in *A*. (*D*) The evoked responses are expressed as fractional suppression of dark current and compared. The three largest responses were recorded with control solution (*A*). For all parts: standard internal solution contained 10 mM EDTA (pCa 10.5, pMg 5.4); V_{hold} = −20 mV; temperature, 16°C; 520-nm light.

intensity from Fig. 2 *A* and *C* are compared in *D*. With 500 μM GTP the mean suppression of dark current by a 2.1 photon per μm^2 flash is 44 ± 0.06% (n = 5), while with 500 μM GDP[βS], a 27-fold brighter flash caused only 22 ± 0.06% (n = 3) suppression of the dark current.

The results obtained with guanine nucleotide analogs support the transduction scheme discussed earlier. The nonhydrolyzable GTP analog, GTP[γS], would be expected to cause permanent activation of $G_{t\alpha}$, resulting in persistent stimulation of PDE and incomplete recovery of the light response. GDP[βS], a GDP derivative that competitively inhibits binding of GTP by G proteins (19), would be expected to hinder the initiation of the transduction process, resulting in reduction of light sensitivity.

It has been hypothesized that one or more inactivation steps of the photoresponse require ATP (20, 21). The upper

part of Fig. 3A shows the effect of ATP on the recovery of responses to bright flashes. The concentration of ATP added to a pipette solution also containing 50 μM cGMP and 20 μM GTP is listed next to each trace. The lower part of Fig. 3A compares the response evoked by less intense flashes in the presence of 0.1, 0.5, and 5 mM ATP. Decreasing the concentration of ATP in the pipette solution slows the kinetics of both the onset and the recovery of the light response. The most dramatic effect of ATP is on the recovery phase of the response. In the absence of ATP, complete recovery of dark current proceeds very slowly, taking up to 40–60 min for bright flashes. There is general agreement between this result and that reported for a similar experiment on truncated rods (9). A principal difference, however, is that we find that ATP accelerates recovery without causing concomitant suppression of light sensitivity. We also find that ATP accelerates the rising phase of the light response, which may indicate that it has an additional function in modulating onset kinetics, possibly through ATP-dependent GTP synthesis.

The requirement of ATP for rapid recovery of photocurrent is consistent with phosphorylation playing a role in the inactivation of an early stage of the transduction process (20–22). Without ATP or some other significant source of phosphate, the inactivation of Rh* occurs at a much lower rate, allowing prolonged activation of subsequent steps in the transduction cascade. As illustrated in Fig. 3B, this can give rise to a stepwise decline in dark current. With each flash there is an increase in the concentration of Rh* causing a new steady-state level of PDE stimulation to be reached as a balance is struck between Rh* activation of G_t and $G_{t\alpha}$·GTP inactivation by GTP hydrolysis. When Rh* inactivation is interfered with by omission of ATP and $G_{t\alpha}$ inactivation is prevented by use of GTP[γS], a steady-state level of PDE stimulation is not reached. Instead, the amount of PDE

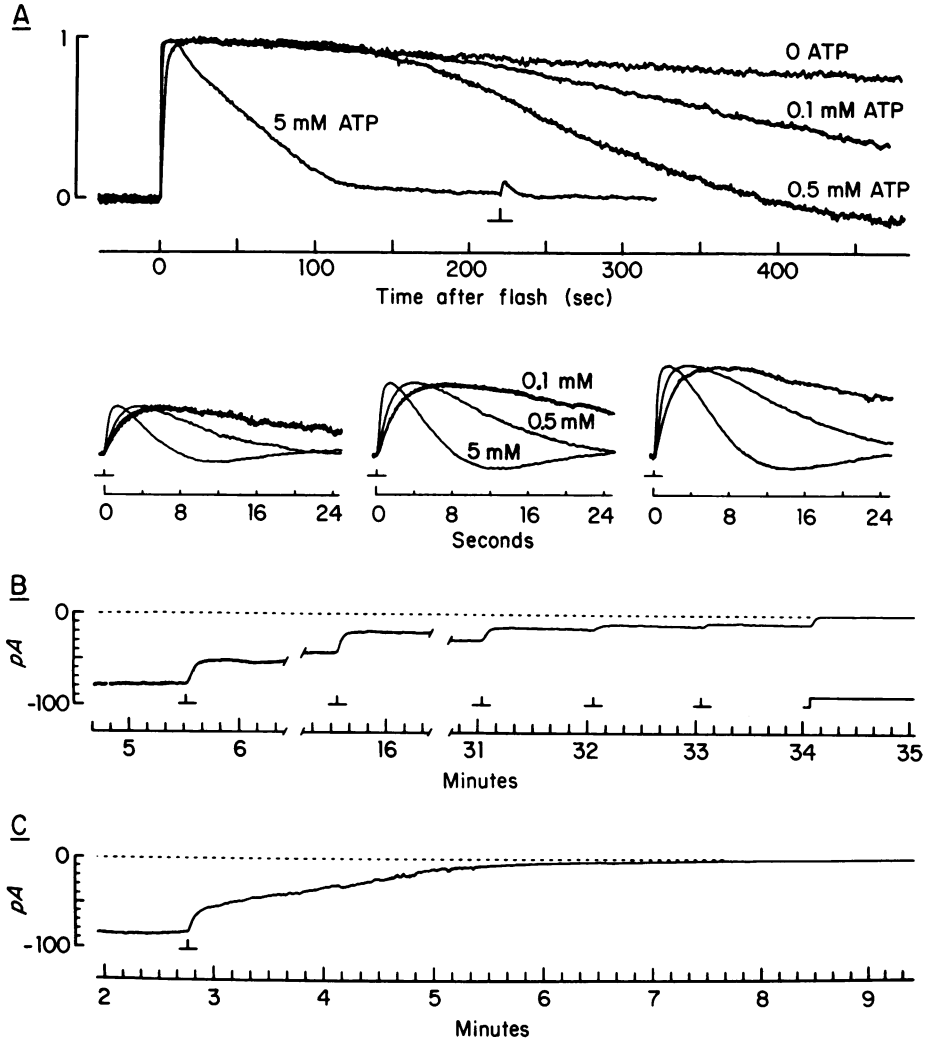

FIG. 3. (A) Effect of ATP on the recovery of the light response. The upper panel shows bright-flash responses recorded with pipette solutions containing 50 μM cGMP, 20 μM GTP, and either 5, 0.5, 0.1, or 0 mM ATP. Responses are normalized to the same peak amplitude, which ranged from 35 to 102 pA corresponding to 94–99% suppression of dark current for all but the zero-ATP response (71% suppression). The influence of ATP on the responses evoked by identical low- to moderate-intensity flashes is shown in the three lower panels. The pipette solution contained 50 μM cGMP, 20 μM GTP, and either 0.1, 0.5, or 5 mM ATP. The response recorded with each solution is scaled to the peak of the 5 mM ATP response and superimposed. The flash intensity in the three panels (left to right) is 5.3, 12, and 122 photons per μm^2. Standard internal solution with 10 mM EDTA (pCa 10.5, pMg 5.4). (B) In absence of ATP sequential flashes give rise to stepwise decline in dark current. Current recording began 3 min after onset of whole-cell recording with a pipette solution containing 50 μM cGMP, 20 μM GTP, and no ATP. Incomplete recovery of responses evoked by a series of identical flashes (21 photons per μm^2) causes staircase suppression of the dark current. The light monitor is shown by line segments below the tracing; horizontal scale indicates time from the onset of whole-cell recording. Breaks in the recording are 8-min, 50-sec and 14-min, 15-sec gaps of darkness. (C) Dark current is fully suppressed by a single flash (2.1 photons per μm^2) when the pipette contains GTP[γS] and no ATP. Standard internal solution contained 50 μM cGMP, 500 μM GTP[γS], no ATP, and 10 mM EDTA (pCa 11.1, pMg 6.25). Light monitor and time are indicated in B. For all parts: temperature, 15–17°C; V_{hold} = −20 mV; 520-nm light.

activated by a single flash continually increases, causing the dark current to decline steadily to zero (Fig. 3C).

Divalent cations were hypothesized to play a major role in earlier models of phototransduction (23). To test their influence, the experiments described above were repeated using internal solutions with concentrations of free Ca^{2+} and Mg^{2+} ranging from 10 mM to 8 pM and 2.2 mM to 0.6 μM, respectively. These changes in divalent cations did not alter the basic results of our experiments. Reducing free Ca^{2+} to 8 pM and Mg^{2+} to 0.6 μM by the addition of 10 mM EDTA caused a large increase in dark current (Table 1) and slowed the kinetics of the light response. Increasing Ca^{2+} to 10 mM with 2.2 mM free Mg^{2+} reduced the dark current and increased the speed of the response. Although large changes in the concentration of divalent cations are able to influence dark current and response kinetics, they do not alter the effect of nucleoside triphosphates on the development and recovery of the light response.

DISCUSSION

We reported previously (10) that detached rod outer segments are able to support a light-sensitive inward dark current during whole-cell recording with pipettes filled with a solution containing a mixture of cGMP, ATP, and GTP. The present experiments describe how the generation of the dark current and the properties of its light sensitivity are influenced by changes in the nucleotide composition of the pipette solution. Our results provide physiological evidence that strongly supports the cGMP-cascade theory of visual transduction.

cGMP is required for dark-current development. In most cases it was supplied by the pipette solution, but detached outer segments possess two other potential sources of cGMP. One is through the synthesis of cGMP via a pathway that requires an exogenous supply of either GTP or ATP. Since the detached preparation lacks an inner segment, this demonstrates unequivocally that the rod outer segment contains guanylate cyclase (24–26). The other source of cGMP is an endogenous pool present in the outer segment at the time of detachment. Unless protected by treatment with a PDE inhibitor, this pool of cGMP declines steadily in darkness, consistent with previous studies reporting a basal level of PDE activity in dark (14, 15). Thus the concentration of cGMP in darkness, and hence the level of dark current, is controlled by the activity of both PDE and guanylate cyclase. To focus on the pathway responsible for light activation of PDE, we attempted to minimize the effect of possible changes in guanylate cyclase activity by using pipette solutions that contained low concentrations of nucleoside triphosphates and provided a constant source of cGMP.

The light sensitivity of detached outer segments is reduced by dialysis with a solution lacking GTP, as well as by solutions containing GDP[βS], a G-protein antagonist. These results are consistent with a large body of biochemical evidence (reviewed in refs. 6–8) that a G protein (G_t) couples light absorption to the stimulation of PDE. Similar biochemical studies showed that termination of the transduction process requires inactivation of two intermediates, Rh* and $G_{t\alpha}$·GTP (7, 8, 20–22). This is accomplished by phosphorylation of Rh* by rhodopsin kinase and hydrolysis of GTP by the intrinsic GTPase activity of $G_{t\alpha}$. In agreement with this mechanism of termination, we find that in the absence of ATP or the presence of GTP[γS], a hydrolysis-resistant GTP analog, a brief flash of light causes persistent activation of PDE.

While our results confirm the major steps in the cyclic-nucleotide hypothesis, questions remain about the finer details of its regulation. This is most apparent with respect to the recovery of the light response. Under a variety of conditions it is possible to identify at least two phases in the recovery of the photocurrent: an early rapid phase, which often undershoots the baseline dark current, and a late slower phase. The relative magnitudes of the two phases vary from cell to cell and typically change over the course of an experiment in a way that accentuates their differences. This can give rise to the appearance of damped oscillations in the recovery of dark current, suggestive of a process controlled by negative feedback. The identity of the feedback signal is not known. One possibility is that changes in intracellular Ca^{2+} govern recovery (27, 28). In our experiments the two phases of recovery were apparent in recordings with solutions containing vastly different amounts of Ca^{2+} (10 mM to 8 pM). This indicates that either the cell has better control over intracellular Ca^{2+} than the experimenter or that Ca^{2+} does not provide the feedback signal that orchestrates recovery.

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